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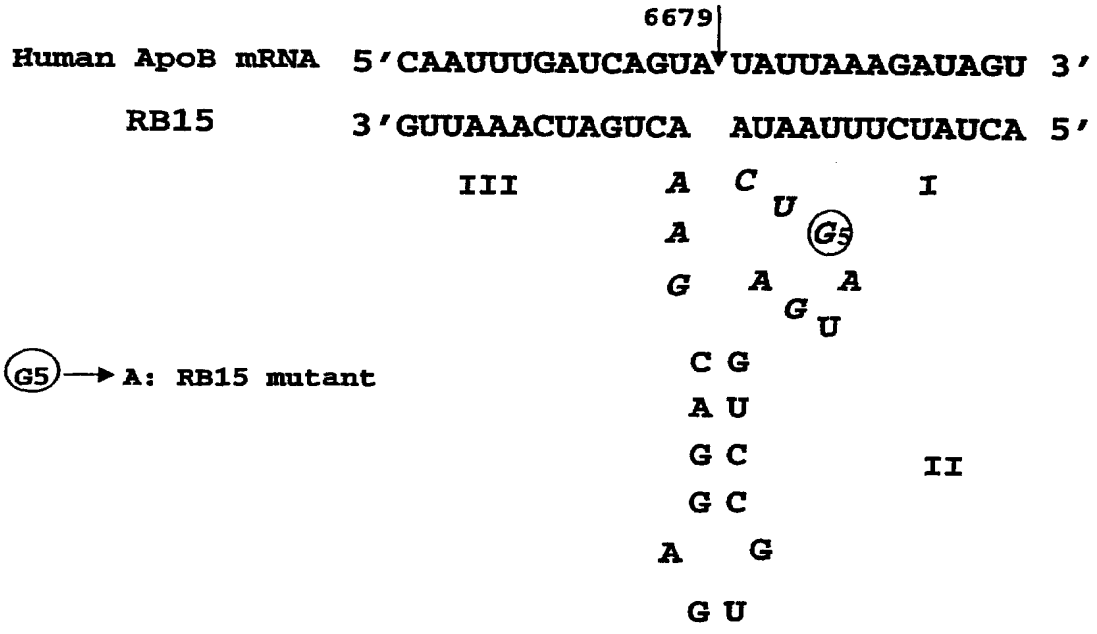
(54) Title: APOLIPOPROTEIN B mRNA-SPECIFIC RIBOZYME



(57) Abstract: The present invention is directed to ribozymes that specifically cleave mRNA for apolipoprotein B. The ribozyme may be used to treat a variety of conditions associated with atherosclerosis, including hypercholesterolemia and hyperlipidemia.

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Apolipoprotein B mRNA-Specific Ribozyme

Field of the Invention

The present invention is directed to an enzymatically active RNA, *i.e.*, a ribozyme, that specifically cleaves the mRNA of apolipoprotein B (apoB). The ribozyme may be encoded by DNA, incorporated into a vector, and used to treat diseases characterized by excessive plasma levels of apoB. In addition, the ribozyme may be used to produce an animal model of hypobetalipoproteinemia.

Background of the Invention

Atherosclerosis is a leading cause of death in the United States. It occurs when blood vessels become progressively occluded due to the formation of plaques within their lumen. This contributes directly to high blood pressure, strokes, heart attacks, and other disorders. One approach that has been taken to the prevention and treatment of atherosclerosis is to use ribozymes that specifically cleave the mRNA transcripts of pathogenic factors. For example, ribozymes have been targeted to the mRNA of apolipoprotein A (U.S. 5,877,022; and 5,599,706) and to the mRNA for cholesterol ester transferase protein (U.S. 5,705,388). Ribozymes of the "hammerhead" class have been found to be particularly effective at cleaving pathogenic transcripts when delivered *in vivo* by means of an adenovirus (Lewin *et al.*, *Nat. Med. 4*:967–971 (1988); *see also* Drenser, *Invest. Opthalmol. Vis. Sci. 39*:681–689 (1988); and Lieber *et al.*, *J. Virol. 70*:3153–3158 (1996)).

A well established risk factor for the development atherosclerotic coronary disease is the presence of an elevated plasma concentration of apoB100 (Sniderman *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 77*:604–608 (1980)). This protein is produced in the liver and, after secretion into the plasma, it forms low density lipoprotein complexes (Breslow, *Physiol. Rev. 68*:85–132 (1988); Kane, *Annu. Rev. Physiol. 45*:637–650 (1983)). Among the conditions associated with an excessive plasma concentration of apoB100 are familial hypercholesterolemia, familial defective apoB100, familial combined hyperlipidemia and hyperapobetalipoproteinemia. The most common of these conditions, familial combined hyperlipidemia, occurs in two percent of the population. A ribozyme capable of specifically cleaving the mRNA of apoB100 would represent a new therapeutic approach to the control of hyperlipidemia and similar diseases .

Summary of the Invention

The present invention is directed to a ribozyme, preferably of the hammerhead motif, that specifically hybridizes to and cleaves apoB100 mRNA. This ribozyme is comprised of three regions. First, there is a stem loop region that is 12 to 40 (and preferably 18–26) bases in length and that assumes a stem loop conformation when the ribozyme hybridizes to apoB100 mRNA. Located within the stem loop region is a target segment three bases long and having the sequence NUX, wherein N represents any base and X is A, C or U. Finally, the ribozyme has two flanking segments 8 to 50 (and preferably 10–20) bases in length. Each of these flanking segments has a sequence that is complementary to a corresponding sequence in apoB100 mRNA. One lies immediately 3', and the other immediately 5', to the stem loop region. The term "complementary" as used in this context means that each nucleotide in the flanking segment of the ribozyme base pairs to its normal partner in apoB100 mRNA, *i.e.*, A base pairs with U and G base pairs with C. Preferably, the ribozyme cleaves apoB mRNA at position 6679, has the target sequence GUA, and has flanking segments comprising sequences consisting essentially of 3'-GUUAAACUAGUCA-5' (SEQ ID NO:1) and 3'-AUAAUUUCUAUCA-5' (SEQ ID NO:2). In its most preferred form, the ribozyme should have a sequence consisting essentially of:

3'-GUUAAACUAGUCAAAGCAGGAGUGCCUGAGUAGUCAUAAUUUCUAUCA-5' (SEQ ID NO:3).

In another aspect, the invention encompasses a DNA molecule consisting essentially of nucleotides encoding the ribozyme described above. This may be part of an expression vector that can be transfected into cells to produce large amounts of the ribozyme. Retroviral vectors will generally be used for *in vivo* delivery, with adenoviral vectors being preferred. The invention encompasses not only the vectors, but also host cells, either *in vitro* or *in vivo*, transformed with the vectors.

The invention is also directed to a method of reducing the concentration of apoB100 in the plasma of a patient by administering an expression vector encoding the apoB-specific ribozyme. Using this method, patients may be treated for hypercholesterolemia, hyperlipidemia, or any other condition in which apoB100 levels are elevated.

In addition, the invention encompasses a mouse administered an expression vector encoding a ribozyme at a concentration sufficient to significantly reduce the plasma concentration of apoB100 relative to that present prior to administration. The term "significantly reduce" means that levels must be decreased to an extent that is significant when analyzed using a scientifically accepted analysis and standard, *e.g.*, $p<0.05$ using Student's T test. Mice treated in this manner may serve as a model of hypobetalipoproteinemia. A transgenic mouse may be used for administration, preferably a transgenic mouse having the *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ phenotype. A transgenic mouse engineered to constitutively express a apoB-specific ribozyme is particularly preferred. Again, the most desirable mouse would have the *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ phenotype.

Brief Description of the Drawings

Figure 1: Figure 1 is a schematic diagram of the human apoB100 mRNA-specific hammerhead ribozyme, RB15. The ribozyme is shown annealed to its corresponding region of apoB mRNA. It can be seen that the ribozyme has a stem loop region (II) flanked by two regions that are complementary to the apoB mRNA sequence (regions I and III). Conserved nucleotides of the catalytic domain are in boldface italic type. When the conserved catalytic nucleotide G^5 is substituted with an A, the ribozyme is designated as the "RB15 mutant." The cleavage target site of apoB mRNA is shown as $GUA^{6679}\downarrow$.

Detailed Description of the Invention

The present invention is based upon the discovery that ribozymes of the hammerhead motif may be developed that specifically cleave apoB100 mRNA. When delivered *in vivo*, this ribozyme causes a substantial reduction in circulating levels of the protein. It therefore may be used to treat or prevent conditions that predispose an individual to the development of atherosclerosis.

Hammerhead ribozymes of the invention are comprised of three distinct regions: a 3-nucleotide target sequence (NUX) where N represents any base and X represents A, C, or U; a conservative catalytic domain; and flanking sequences (typically 10 to 30 bases in length) with sequences complementary to those of the apoB100 mRNA. The most preferred ribozyme is RB15 and is shown in Figure 1. This has a stem loop region 22 nucleotides in length that is flanked by two 13 nucleotide segments that anneal to apoB100 mRNA. Using this ribozyme, cleavage occurs at position 6679.

Other cleavage sites may be selected using the method described by McSwiggen (U.S. 5,525,468) and regions containing the sites may be targeted by changing the portions of the ribozyme undergoing hybridization. Guidance in designing the ribozymes may be obtained in a number of references that have reported the complete cDNA sequence for human apoB100 (Knot, *et al.*, *Nature 323*:734 (1986); Knot, *et al.*, *Nucl. Acid Res. 14*:7501-7503 (1986); Deeb, *et al.*, *Proc. Natl. Acad. Sci. USA 83*:419-422 (1986); Chen, *et al.*, *J. Biol. Chem 261*:12918-12921 (1986); *see also* Gen Bank Accession No. X04506).

Methods for optimizing the activity of ribozymes have been reported in the art and may be used in conjunction with the present invention. For example, ribozyme activity may be altered by changing the length of the flanking segments binding to the substrate RNA or by chemically synthesizing ribozymes with modifications that prevent degradation by serum ribonucleases (*see* WO 92/07065; Perrault, *et al.*, *Nature 344*:565 (1990); Pieken, *et al.*, *Science 253*:314 (1991)). In addition, chemical modifications can be made to the sugar moieties of enzymatic RNA molecules.

Hammerhead ribozymes of the invention and in particular RB15, can be made by any method known in the art. In general, it will be most convenient for the ribozymes to be chemically synthesized using procedures such as that described by Usman, *et al. (J. Am. Chem. Soc. 109*:7845-7854 (1987); and Scaringe, *et al.* (*Nucl. Acid Resp. 18*:5433-5441 (1990)). A preferred method for making pB15 is described in the Example section herein. Once synthesized, ribozymes can, if desired, be modified to enhance their stability by incorporating 2'-O-methyl groups. The ribozymes may be purified by gel electrophoresis or purified by high pressure liquid chromatography.

Although ribozymes may be administered directly to cells or injected into an animal, greater effectiveness will generally be achieved by using DNA sequences encoding the ribozyme. This DNA may then be incorporated into an expression vector and delivered to cells either *in vitro* or *in vivo*. The effectiveness of ribozymes produced in this manner has been demonstrated in a number of different reports (*see e.g.* Chen, *et al.*, *Nucl. Acid. Resp. 20*:4581-4589 (1992); Yu, *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 1990*:6340-6344 (1993)). Among the vectors that may be used with apoB100 specific ribozymes are plasmid DNA vectors, viral DNA vectors or, alternatively, viral RNA vectors. A preferred adenoviral vector is described in the Examples section herein.

Ribozymes may be administered to cells by a variety of methods known in the art, *e.g.*, by encapsulation in liposomes, by iontophoresis or by incorporation into other vehicles such as hydrogels, psyclodexdrins, or microspheres. When used as an *in vivo* treatment method, it is preferred that DNA encoding ribozyme be delivered by means of an adenoviral vector. Delivery to a patient may be made by directed injection, or by use of a catheter, infusion pump or stent. Alternative routes of delivery include, but are not limited to, intravenous injection, intramuscular injection, subcutaneous injection, inhalation, oral delivery, topical delivery, systemic delivery, or intraperitoneal delivery. The effectiveness of a given treatment may be ascertained by determining plasma apoB100 levels using any convenient assay, *e.g.* an agglutination assay (U.S. 5,180,679) or an enzyme immunoassay (U.S. 4,722,893). Specific assays suitable for use are provided under the Examples.

As noted previously, RB15 may be used as a therapeutic agent in treating patients having conditions characterized by elevated apoB100 levels. For example, an adenovirus vector encoding RB15 may be used to treat patients for hypercholesterolemia or hyperlipidemia. The total dosage of ribozyme that should be administered to a patient should be at least an amount sufficient to cause a significant reduction in the patient's plasma levels of apoB100. This may be determined by measuring apoB100 directly or by measuring other related factors such as cholesterol levels. Generally, a patient should be given a relatively low dose initially, with increases being made as necessary. Dosage for an individual patient will be determined by an attending physician based on clinical conditions using methods well known in the art. The therapeutic ribozyme may be used either alone, or in conjunction with other agents appropriate to a patient's condition, *e.g.* other drugs used to lower cholesterol levels.

In another aspect, the invention is directed to a mouse administered a vector comprising an apoB-specific ribozyme (preferably RB15) at a concentration sufficient to reduce plasma apoB levels. Such mice may serve as a model of hypobetalipoproteinemia both to study the effects of this condition and in assays designed to identify agents that may offset its effects. As disclosed in the Examples section, the mice are preferably transgenic animals engineered to have the *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ phenotype. Methods for making these mice are described under Examples (*see also* L'Huillier *et al.*, *Proc. Natl. Acad. Sci. USA 93*:6698–6703 (1996) and Larsson *et al.*, *Nucl. Acids Res. 22*:2242–2248 (1994)). Animals having this phenotype may undergo further engineering to constitutively make RB15.

Examples

Example I: ApoB mRNA-Specific Hammerhead Ribozyme

In this study, we designed hammerhead ribozymes targeted at apoB100 mRNA sequences of $GUA^{6679}\downarrow$ and $AUA^{6665}\downarrow$, flanking the edited base C^{6666}. The study demonstrates that the hammerhead ribozyme cleaves at the precise target site and decreases the levels of apoB mRNA. Furthermore, ribozyme cleavage produces a truncated protein of the expected size.

A. Methods

Construction of Plasmid Vector

ApoB mRNA-Specific Hammerhead Ribozymes

Oligonucleotides used for engineering apoB mRNA-specific hammerhead ribozymes were synthesized using standard techniques and are shown in Table 1. Briefly, sense and antisense strands of oligonucleotides of apoB mRNA-specific hammerhead ribozyme were annealed and cloned into *Xba*I and *Cla*I sites of a pGem 7Zf(+) vector (Promega, Madison, WI). Each construct was sequenced using Sequenase II (Amersham Pharmacia Biotech) in the presence of single strand DNA-binding protein (Amersham Pharmacia Biotech). Constructs of target sequences of $AUA^{6665}\downarrow$ and $GUA^{6679}\downarrow$ are designated pRB16 and pRB15, respectively. A point mutant of the conserved catalytic domain at nucleotide $G^5 \rightarrow A$ (G5A) of the RB15 construct is designated as "the pRB15 mutant," and the numbering of the conserved catalytic domain is according to Hertel *et al.* (*Nucleic Acids Res. 20*:3252 (1992)). The sequences of ribozymes RB15 and RB16 are available in GenBank™. The numbering of apoB corresponds to the published human apoB100 sequence (Knott *et al.*, *Nucleic Acids Res. 14*:7501–7503 (1986)) (GenBank™ accession number X04506).

Table 1: Oligonucleotides

Ribozyme	Sequence
RB 15 mutant (top strand)	5'-CTCTAGAACTATCTTTAATA*CTAATGAGTCCGTGAGGACGAA***AC** TGATCAAATTGATCGATC-3' (SEQ ID NO:4)
RB15 mutant (bottom strand)	5'-GATCGATCAATTTGATCA**GT***TTCGTCCTCACGGACTCATTAG*TA TTAAAGATAGTTCTAGAG-'3 (SEQ ID NO:5)
RB15 (top strand)	5'-CTCTAGAACTATCTTTAATA*CTGATGAGTCCGTGAGGACGAA***AC** TGATCAAATTGATCGATC-3' (SEQ ID NO:6)

RB15 (bottom strand)	5'-GATCGATCAATTTGATCA**GT***TTCGTCCTCACGGACTCATCAG*TA TTAAAGATAGTTCTAGAG-3' (SEQ ID NO:7)
RB16 (top strand)	5'-CTCTAGACTGATCAAATTG*CTGATGAGTCCGTGAGGACGAAA***AT** CATATATGTCATCGATC-3' (SEQ ID NO:8)
RB16 (bottom strand)	5'-GATCGATGACATATATG**AT***TTCGTCCTCACGGACTCATCAG*CAA TTTGATCAGTCTAGAG-3' (SEQ ID NO:9)

Restriction enzyme sites are underlined.
Mutant nucleotide and targeted sites are in bold.
Conserved sequences of ribozyme cleavage cassette are in italics.

U6 snRNA Construct

A DNA fragment of U6 snRNA (110 base pairs) was cloned into pGem7Zf(+) (pU6) and used to generate a probe for the quantitation of U6 snRNA transcripts by an RNase protection assay. The anti-U6 snRNA probe of 143 nucleotides was produced by *Eco*RI-linearized pU6 using SP6 RNA polymerase.

Human ApoB Construct

pB3, containing a human apoB cDNA fragment of nucleotides 4953–5602 in pGem7Zf(+), was used to generate the antisense apoB probe for the quantitation of apoB transcripts by the RNase protection assay. The anti-B3 probe of 721 nucleotides was produced by *Xba*I-linearized pB3 using T7 RNA polymerase.

In Vitro Transcription Reaction

For synthesis of *in vitro* transcripts, a maxiscript kit from Ambion (Austin, TX) was used. Briefly, a linearized DNA template (2 μg) was incubated in 20 μl of transcription buffer containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 20 mM dithiothreitol, 5 mM ATP, 5 mM CTP, 5 mM GTP, 5 mM UTP, 40 units of RNase inhibitor, and 10 units of RNA polymerase. The reaction was carried out at 37°C for 1–2 h, DNA template was removed by incubation with 2 units of RNase-free DNase I at 37°C for 30 min, and the RNA was recovered after phenol/chloroform extraction, followed by ethanol precipitation with ammonium acetate. For synthesis of the radiolabeled transcript, 50 μCi of [^{32}P]UTP (10 mCi/ml, Amersham Pharmacia Biotech) was used instead of UTP. At the end of the

reaction, the free nucleotides were removed using the nick column method (Amersham Pharmacia Biotech).

In Vitro Ribozyme Cleavage Reaction

Control ribozyme RNA corresponding to sense apoB RNA was synthesized from the ribozyme plasmid vectors pRB15 and pRB16 (*Xba*I-linearized) using T7 RNA polymerase. Synthetic ribozyme RNA corresponding to antisense apoB RNA (*Hin*dIII-linearized) was produced using Sp6 RNA polymerase. The concentration of synthetic ribozyme RNA was determined by measuring optical density at 260 nm. pGem-CAA, containing a human apoB cDNA fragment (nucleotides 6506–7335) (Teng, *et al.*, *J. Biol. Chem. 265*:20616–20620 (1990)), was used to transcribe a ^{32}P-radiolabeled 829-nucleotide synthetic apoB RNA (pGem-CAA linearized with *Hin*dIII) using T7 RNA polymerase in the presence of [^{32}P]UTP (10 mCi/ml, Amersham Pharmacia Biotech). The *in vitro* ribozyme cleavage reaction was performed using 1 x 10^5cpm ^{32}P-labeled apoB RNA as substrate and 2 μg of ribozyme RNA in a buffer containing 50 mM Tris, pH 7.5, 20 mM $MgCl_2$, and 1 mM EDTA. The reaction was carried out for 1–2 h at either 37 or 50°C as indicated. The products were analyzed using 5% polyacrylamide urea gel electrophoresis. The gel was autoradiographed and quantitated using a PhosphorImager SF scanner (Molecular Dynamics, Inc., Sunnyvale, CA).

Reverse Ligation-Mediated PCR (RL-PCR)

Synthesis of the RNA Linker

The following two synthetic oligonucleotides were annealed and used for synthesis of the RNA linker using T7 RNA polymerase: bottom, 5′-TTTCAGCGAGGGTCAGCCTATGCCCTATAGTGAGTCGTATTA (SEQ ID NO:10); top, 5′-TAATACGACTCACTATAG (SEQ ID NO:11). The synthesized RNA linker was used to ligate the phosphorylated apoB mRNA after ribozyme cleavage.

DNA Primers

Three DNA primers 3′ of the RB15 ribozyme cleavage site GUA^{6679}↓ were selected, as suggested by Bertrand *et al.* (*Methods Mol. Biol.*, (Turner, P. C., ed) vol. 74, pp. 311–323, Humana Press Inc., Totowa, NJ). Primer 1 (P1, 5′-TCAATGATTTCATCAATAATA, SEQ ID

NO:12), corresponding to human apoB cDNA nucleotides 6728–6748, was used for reverse transcription. Primer 2 (P2, 5′-AGCTATTTTCAAATCATGTAA, SEQ ID NO:13), corresponding to human apoB cDNA nucleotides 6699–6719, was used as the downstream primer for PCR amplification. Primer 4 (P4, 5′-CAAATCATGTAAATCATAAC, SEQ ID NO:14), corresponding to human apoB cDNA nucleotides 6691–6711, was used for sequencing.

RL-PCR

Total cellular RNA (0.7 mg) after ribozyme cleavage was phosphorylated at the 5′-OH end using T4 polynucleotide kinase (Amerisham Pharmacia Biotech). The phosphorylated RNA was ligated with RNA linker (100 μg/ml) using T4 RNA ligase (Roche Molecular Biochemicals). After ligation, cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) with the P1 primer, followed by PCR using the downstream primer (P2) and the upstream primer (DNA linker primer, 5′-GGGCATAGGCTGACCCTCGCT, SEQ ID NO:15) that is complementary to the RNA linker. This PCR product can be detected either by using ^{32}P-end-labeled primer P2 or by ethidium bromide visualization after analysis with 8% polyacrylamide-urea gel electrophoresis.

Sequencing

Nonradioactive primers, P2 and DNA linker primer, were used to produce the PCR product for sequencing. After amplification, the PCR product was treated with 15 units of exonuclease (10 units/μl) and 3 units of shrimp alkaline phosphatase (2 units/μl) to remove the primers and free nucleotides. The purified PCR product was sequenced using the sequencing primer P4 with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech). The sequenced product was analyzed on 8% polyacrylamide-urea gel and detected by autoradiogram.

Construction of Adenoviral Ribozyme Vectors

Construction of Recombinant Adenoviral Ribozyme Vectors

ApoB-specific hammerhead ribozymes, RB15, RB16, and RB15 mutant, were cloned into the adenoviral shuttle vector, pAvS6, which contains a Rous sarcoma virus promoter as described by Teng, *et al., J. Biol. Chem. 269*:29395–29404 (1994). The recombinant adenovirus was prepared

by co-transfection of pAvS6 containing apoB-specific hammerhead ribozyme and pJM17 into 293 cells. Adenoviral vectors containing apoB-specific ribozymes were plaque-purified on these cells. High titer recombinant adenovirus was amplified on 293 cells and purified by CsCl gradient centrifugation as described previously (Teng *et al., J. Biol. Chem. 269*:29395–29404 (1994)). Recombinant adenovirus Av1LacZ4 was supplied by Genetic Therapy Inc. (Gaithersburg, MD). It has the same structure as AvRB15 except that it contains a 3.1-kb nuclear targeted β-galactosidase cDNA insert instead of ribozyme.

Cell Culture and Recombinant Adenovirus Infection

Human hepatoma cell line (HepG2) was cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum, 2 mg/ml glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids at 37°C with 5% CO_2. HepG2 cells were plated onto six-well culture dishes until the cells reached 80% confluency. The cells were infected with 2 x 10^5 pfu of AvRB15, AvRB16, and AvRB15 mutant as indicated in each experiment.

Quantitation and Characterization of RB15 and ApoB

Quantitation of the Levels of Ribozyme RB15 RNA and ApoB mRNA After AvRB15 Infection

HepG2 cells were plated as described above. The cells were infected with 2 x 10^5 pfu of AvRB15 for 5, 10, 15, and 24 h. At each time point, RNA was extracted from cells using an Ultraspec RNA kit (Biotecx Laboratory Inc., Houston, TX). The expression levels of RB15 RNA and apoB mRNA in the cells were quantitated using an RNase protection RPA II kit (Ambion). Briefly, an RB15 RNA probe of 102 nucleotides was produced from the pRB15 vector (linearized with *Xba*I) using Sp6 RNA polymerase in the presence of [^{32}P]UTP (Amersham Pharmacia Biotech). The RNase protection assay was carried out with 10 μg of total RNA and 3 x 10^4 cpm of probe in 20 μl of hybridization buffer. The mixture was incubated at 45°C overnight. At the end of this reaction, the mixture was treated with 100 μl of RNase digestion buffer containing RNase A and RNase T1 for 30 min at 37°C. The protected RNA fragment (59 nucleotides) was precipitated and analyzed with 8% polyacrylamide-urea gel electrophoresis and quantitated using a PhosphorImager SF scanner.

To determine the levels of apoB mRNA after AvRB15 infection, a ^{32}P-labeled antisense apoB RNA probe of 721 nucleotides was produced from the pB3 vector (linearized with *Xba*I) using T7 RNA polymerase in the presence of [^{32}P]UTP (10 mCi/ml, Amersham Pharmacia Biotech). The RNase protection assay was performed as described above. After RNase digestion, the protected fragment of 640 nucleotides was analyzed with 5% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner.

Quantitation of the Levels of RB15 RNA in Nucleus and Cytoplasm Fractions After AvRB15 Infection

Cytoplasmic RNA Extraction–HepG2 cells were plated onto 10-cm culture dishes and infected with 5 x 10^5 pfu of AvRB15 for 15 h. Cytoplasmic RNAs were extracted as described by Bertrand *et al.* (*RNA 3*:75–88 (1997)). Briefly, the cells were pelleted by centrifugation at 1000 rpm for 5 min, rinsed with ice-cold PBS, and resuspended in RNA extraction buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.0, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 40 units of RNasin). The samples were placed on ice for 5 min, and centrifuged at 12,000 x *g* for 90 s. The supernatant was treated at 37° C for 30 min with proteinase K (50 μg/ml) in a digestion buffer containing 0.2 M Tris, pH 8.0, 25 mM EDTA, 0.3 M NaCl, and 2% SDS. Cytoplasmic RNA was extracted using phenol/chloroform and precipitated with ethanol.

Nuclear RNA Extraction– After AvRB15 infection, the cells were washed twice with ice-cold PBS and resuspended in buffer H (15 mM NaCl, 60 mM KCl, 1 mM EDTA, 10 mM Tris, pH 7.5, 0.2% Nonidet P-40, and 5% sucrose. The cells were then homogenized using a Dounce homogenizer (pestle A, six gentle strokes) to release the nuclei. The nuclei were purified by centrifugation through a 10% sucrose cushion (buffer H without Nonidet P-40, containing 10% sucrose). Nuclear RNA was extrcated using phenol/chloroform and precipitated with ethanol.

Quantitation of RB15 RNA and U6 snRNA by RNase Protection Assay

Cytoplasmic RNAs (10 μg) and nuclear RNAs (10 μg), prepared as described above, were used to quantitate RB15 RNA and U6 snRNA by RNase protection assay (Ambion). The method for the quantitation of RB15 RNA has been described above. To measure the levels of U6 snRNA, a ^{32}P-labeled anti-U6 snRNA probe of 143 nucleotides was produced from the pU6 vector (linearized with

*Eco*RI) using Sp6 RNA polymerase in the presence of [^{32}P]UTP. After RNase digestion, a protected fragment of 110 nucleotides was analyzed with 6% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner. The distributions of nucleocytoplasmic RB15 RNA or U6 snRNA were expressed as a percentage of the total amount of RB15 or U6 RNAs in the fraction of cytoplasm plus nucleus.

Continuous Labeling or Pulse-Chase Labeling Experiment

HepG2 cells were plated onto six-well plates until 80% confluent. Cells were infected with 2×10^5 pfu of AvRB15 or AvRB15 mutant in serum-free EMEM media for 15 h. Control cells were without adenovirus infection. The cells were washed with PBS and methionine-free EMEM (ICN Biomedicals Inc.), followed by incubation with methionine-free EMEM for 30 min. The cells were then labeled with a Tran^{35}S-label (100 μCi/well, ICN, Costa Mesa, CA) in methionine-free EMEM media. For the continuous-labeling experiment, the cells were labeled at 37°C for 0, 10, 30, 45, 60, 120, and 180 min. For pulse-chase experiments, the cells were labeled for 15 min at 37°C. After pulse labeling, the media were removed, and the cells were incubated with serum-free EMEM containing 2mM methionine for 0, 10, 30, 45, 60, 120, and 180 min. At each time point, medium was collected, and cells were lysed with 2 ml of buffer (50 mM Tris, pH 9.0, 100 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor mixture (Roche Molecular Biochemicals). Both cellular and medium samples were subjected to immunoprecipitation using human apoB monoclonal antibody 1D1 or 4G3 (Lipoproteins and Atherosclerosis Group, University of Ottawa Heart Institute, Ottawa, Canada).

Immunoprecipitation of ApoB

Briefly, monoclonal antibody 1D1 or 4G3 was incubated with protein A-Sepharose (RepliGen) in binding buffer (1.5 M glycine, 3.0 M NaCl, pH 8.9) for 1 h. The beads were then blocked with 10% nonfat dry milk to reduce nonspecific background binding. Immunoprecipitation was carried out by incubating 1 ml of medium or cell lysate with antibody-protein A complex at 4°C overnight. Immunocomplex beads were washed twice with wash buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), once with a 1:1 mixture of wash buffer and 1 M NaCl, and once with wash buffer only. The beads were suspended in a sample buffer containing 8 M urea and 2% SDS. ApoB proteins were resolved on 6% ProSieve 50 gel (FMC, Rockland,

ME). The gel was fixed and enhanced with fluorography. The migration bands of apoB100 and truncated apoB were quantitated using the PhosphorImager SF scanner.

Characterization of Secreted Apolipoprotein B-Containing Lipoproteins

In some experiments, after the pulse-chase experiment, the media were subjected to sequential ultracentrifugation at densities of 1.006, 1.063, and 1.210 g/ml. The salt density of the media was adjusted by adding appropriate amounts of NaCl/KBr salt solutions. After centrifugation, lipoproteins were collected and dialyzed extensively. Each lipoprotein fraction was immunoprecipitated with the monoclonal antibody 1D1 or 4G3. The method for immunoprecipitation of apoB was the same as described above.

B. Results

Hammerhead Ribozymes Targeted at Sites Flanking C^{6666} of ApoB mRNA

A schematic diagram of the hammerhead ribozyme targeted at $GUA^{6679}\downarrow$ of apoB mRNA is shown in Fig. 1. The conserved catalytic domain (stem II) is flanked by 13 nucleotides complementary to the apoB mRNA targeted at $GUA^{6679}\downarrow$ (stems I and III). The nucleotide sequences in *boldface type* are essential nucleotides required for ribozyme cleaving activity. Mutation of these nucleotides will result in partial or complete loss of ribozyme function. The RB15 mutant was constructed by substituting the G^5 with an A (G5A). *In vitro* ribozyme activity was assayed using ribozyme RNAs of RB15, RB16, and RB15 mutant. ApoB RNA of 829 nucleotides was cleaved by ribozyme RB15 to produce two fragments of 656 and 173 nucleotides, whereas cleaving by ribozyme RB16 generated two fragments of 670 and 159 nucleotides. Ribozyme RB15 RNA targeted at sequences $GUA^{6679}\downarrow$ cleaved 49±6.6% (n=5) of apoB RNA at 37°C in 1 h. As expected, the proportion of cleaved apoB RNA increased to 91±4.1% (n-5) at 50°C. Ribozyme RB16 RNA targeted at sequences $AUA^{6665}\downarrow$ cleaved apoB RNA more efficiently than ribozyme RB15. At 37°C for 1 h, it cleaved 66±12% (n-5) of apoB RNA; the proportion of cleaved apoB RNA increased to 90±2.9% (n-5) at 50°C. Ribozyme cleaving activity was time-dependent. The activity of both target sites (GUA↓ and AUA↓) at 37°C after 2-h incubation increased to 75 and 86%, respectively. The cleaving activities of both RB15 and RB16 reached maximum levels (>95%) at 50°C after a 2-h incubation. Control experiments using either antisense ribozyme RB15 RNA or ribozyme RB15 mutant RNA had no detectable cleaving activity.

Effect of Adenovirus-Mediated Ribozyme Expression in HepG2 Cells

To examine apoB mRNA-specific ribozyme activity in cells, ribozymes targeted at nucleotides 6665 (AUA↓) and 6679 (GUA↓) were used to construct the recombinant adenoviral vectors, AvRB16 and AvRB15, respectively. AvRB15 mutant was produced as the control for inactive ribozyme RB15.

Ribozyme RB15 Gene Expression in HepG2 Cells

HepG2 cells were infected with 2 x 10^5 pfu of AvRB15 for 5, 10, 15, and 24 h. At each time point, total RNA was extracted from cells, and RB15 RNA expression was determined by the RNase protection assay. The expression of RB15 was time-dependent, increasing from 12±4.6 (n=4) to 21±4.4 (n=4) and 98±27 (n=4) pg of RB15 RNA/10 μg of total RNA at 5, 10, and 15 h, respectively. By 24 h, RB15 expression increased to 3090±147 pg of RB15 RNA/10 μg of total RNA. This markedly increased gene expression was probably the result of adenovirus replication, since HepG2 cells contain E1A-like proteins (Spergel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* *88*:6472–6476 (1991)). All of the experiments described in this study were performed by infection of HepG2 cells with recombinant ribozyme adenovirus of 15 h.

Detection of 3′ Ribozyme Cleavage Product in HepG2 Cells Using RL-PCR

Many investigators have reported that ribozyme cleavage products cannot be detected by classical techniques, such as Northern blot analysis or RNase protection assay. Bertrand *et al.* (*Meth. Mol. Biol.* (Turner, P.C., ed) vol. 74, pp. 311–323, Humana Press Inc., Totowa, NJ) developed a sensitive RL-PCR method that can detect the 3′ cleavage product after ribozyme reaction. Using this technique, we were able to detect the 3′ ribozyme cleavage product of apoB mRNA after AvRB15 treatment in HepG2 cells. We confirmed the precise cleavage site in apoB mRNA by direct sequencing. After AvRB15 treatment, a radioactive band of 65 nucleotides was detected only in the RNA that was ligated with RNA linker, transcribed with reverse transcriptase, and followed by PCR. There was no detectable band when the RNA was not ligated to RNA linker or not transcribed with the addition of reverse transcriptase. Control RNAs from Av1LacZ-treated or AvRB15 mutant-treated cells after RL-PCR did not have any detectable bands. To confirm the exact cleavage site, we sequenced the PCR product. RB15 ribozyme cleaved human apoB mRNA precisely at the expected position of nucleotide 6679 in HepG2 cells.

hammerhead ribozymes cleave apoB mRNA in HepG2 cells, resulting in the production of a truncated protein that is secreted into the media.

The media from controls (nontreated and AvRB15 mutant-infected cells), AvRB15-infected cells, and AvRB16-infected cells were fractionated by sequential ultracentrifugation into VLDL (d<1.006 g/ml), LDL (d=1.0006–1.063 g/ml), and HDL (d=1.063–1.210 g/ml), followed by immunoprecipitation with monoclonal antibody 1D1. In nontreated HepG2 cells, only apoB100 was detected in VLDL, LDL, and HDL fractions. The same results were obtained from AvRB15 mutant-infected cells. In contrast, after AvRB15 treatment, a truncated apoB was detected in the HDL fraction, but not in the fractions of VLDL or LDL. Similarly, the truncated apoB band was detected, but barely visible in the HDL fraction of cells treated with AvRB16. Therefore, the result suggests that the truncated apoB produced after apoB mRNA-specific ribozyme treatment in HepG2 cells was assembled and secreted as HDL-like lipoprotein particles. As noted, a band of ~120 kDa was observed in LDL and HDL fractions of samples treated with AvRB15 and AvRB16. The nature of this band is not clear. Interestingly, unlike the result demonstrated with the *in vitro* ribozyme cleavage experiment, under *in vivo* conditions, RB15 ribozyme targeted at $GUA^{6679}\downarrow$ cleaved apoB mRNA more efficiently than RB16 ribozyme targeted at $AUA^{6665}\downarrow$.

We expected Rous sarcoma virus-driven RNA to be localized in the cytoplasm. However, results indicated that apoB mRNA-specific ribozyme cleavage produced a truncated apoB, which was secreted as HDL particles. This is unusual, and it was necessary to confirm the location of the expressing ribozyme RNA. RNAs from the nucleus and cytoplasm fractions were extracted from HepG2 cells after AvRB15 treatment. The RNase protection assay was used to quantitate the distribution of ribozyme RB15 RNA in each fraction. To monitor leakage of nuclear contents into the cytoplasm fraction, we measured endogenous U6 snRNA, which is expected to be located in the nucleus only (Terns *et al.*, *Genes Dev.* 7:1898–1908 (1993)). The results showed that 80±6.3% (n=4) of U6 snRNA was found in the nuclear fraction, whereas 85±3.5% (n=4) of RB15 RNA was in the cytoplasm fraction. Therefore, by normalizing against the amount of U6 snRNA that leaked into the cytoplasm, we estimated that the relative amount of RB15 RNA in the cytoplasm was ~70%.

Effect of AvRB15 Treatment on the Levels of ApoB mRNA in HepG2 Cells

To determine whether AvRB15 treatment has an effect on the levels of apoB mRNA, we used the RNase protection assay to quantitate apoB mRNA concentration after treatment. A protected fragment of 640 nucleotides was introduced into nontreated HepG2 cells and into cells treated with either AvRB15 mutant or AvRB15. The levels of apoB mRNA decreased ~80% (ratio of AvRB15-treated/non-treated RNA, 0.200±0.014, n=3) after AvRB15 treatment, compared with that of nontreated HepG2 cells (1.0, n=3). In contrast, there was no effect on apoB mRNA levels of HepG2 cells treated with AvRB15 mutant (1.015±0.115, n=3). Human GAPDH transcript was measured and used as an internal control for the assay. There was no change in the levels of the consecutively expressed GAPDH transcripts in nontreated HepG2 cells (1.0, n=3) or cells treated with either AvRB15 mutant (1.004±0.035, n=3) or AvRB15 (0.91±0.091, n=3). Therefore, the apoB-specific hammerhead ribozyme greatly reduced apoB mRNA transcripts with high specificity.

Effect of AvRB15 and AvRB16 on ApoB Biosynthesis and Secretion in HepG2 Cells

Next, we investigated the effect of AvRB15 and AvRB16 on apoB biosynthesis and secretion in HepG2 cells. HepG2 cells were infected with AvRB15 or AvRB16 (2×10^5 pfu) for 15 h. Cells infected with Av1LacZ4 and AvRB15 mutant (2×10^5 pfu) were used a controls. After infection, cells were washed and labeled with [^{35}S]methionine for 15 min and chased for 3 h. At the end of incubation, culture media and cell lysates were immunoprecipitated using human apoB-specific monoclonal antibody 1D1 (which recognizes residues 474–539). Media from nontreated and Av1LacZ-infected HepG2 cells contained apoB100 only. The same result was obtained from AvRB15 mutant-infected cells. In contrast, media from AvRB15- and AvRB16-treated cells had apoB100 and a truncated apoB of the expected molecular weight. There was substantially more truncated apoB in the media from cells treated with AvRB15 (~50% of total secreted apoB) than cells treated with AvRB16 (~5% of total secreted apoB). Similarly, in cell lysates, apoB100 was the only protein detected in nontreated and Av1LacZ4-treated cells, whereas both apoB-100 and a truncated apoB were detected in AvRB15- or AvRB16-treated cells. To confirm that the detection of truncated apoB was not the result of apoB degradation, culture media and cell lysates were immunoprecipitated using the human apoB-specific monoclonal antibody 4G3 (the C-terminal region-specific antibody that recognizes residues 2980–3084). Only apoB100 was detected in nontreated, Av1LacZ4-, AvRB15-, or AvRB16-treated cells. Therefore, the results suggest that apoB-specific

Kinetics of ApoB100 and Truncated ApoB in HepG2 Cells After AvRB15 Treatment

To understand more about the physiological effect of AvRB15 on the synthesis and secretion of apoB100 and truncated apoB in HepG2 cells under the supplement of oleic acid, we carried out the following studies.

Continuous Labeling of HepG2 Cells After AvRB15 Treatment with [^{35}S]Methionine— HepG2 cells cultured in the presence of 3% BSA or 1 mM oleic acid/BSA (Sigma), were infected with AvRB15 for 15 h. After infection, cells were labeled with [^{35}S]methionine, and cell media and lysates were collected at 0, 10, 30, 45, 60, 120, and 180 min. ApoB was immunoprecipitated with monoclonal antibody 1D1 and analyzed by SDS-PAGE. After AvRB15 treatment, both apoB100 and truncated apoB were synthesized in the cells and secreted into the media. In both conditions the synthesis rate of truncated apoB was faster than that of apoB100 (the unit of the result is expressed as PhosphorImager counts (*PI*)/2 h/mg of cell protein. Under BSA conditions, truncated apoB was 151,640 and apoB100 was 36,351 and under oleate/BSA conditions, truncated apoB was 340,020 and apoB100 was 224,983. In contrast, for both conditions there were more apoB100 molecules secreted into the media compared with truncated apoB. In the presence of oleate, 5-fold more full-length apoB100 molecules were secreted than truncated apoB. When we estimated the percentage of secreted radiolabeled apoB to that synthesized in the cells, only ~5% of the radiolabeled truncated apoB was secreted into media compared with that of apoB100 (~20%). Therefore, the result suggests that compared with apoB100, only a small amount of the truncated apoB was secreted.

Pulse-Chase Experiments— To confirm that the synthesis of truncated apoB is not derived from post-translational degradation of apoB100, we examined protein synthesis after AvRB15 treatment with a 15-min pulse-labeling followed by a chase of 10, 30, 45, 60, 120, and 180 min in the presence of 1 mM oleate/BSA. ApoB was immunoprecipitated and analyzed from cell media and lysates. As observed in the continuous labeling experiment, after AvRB15 treatment, the cells secreted relatively more full-length apoB100 into the media than truncated apoB. During the first 60 min after the chase, in the presence of oleate, the amount of labeled intracellular apoB100 and truncated apoB decreased by ~80%. For apoB100, most of the radioactivity (>70%) was recovered in the media, whereas only

~30% of truncated apoB radioactivity was recovered in the media. These results suggest that, compared with full-length apoB100, a substantial amount of the truncated apoB was degraded intracellularly.

C. Discussion

ApoB100 is the major protein component of LDL and is responsible for the binding of this lipoprotein to the LDL receptor. Studies in humans indicate that overproduction of apoB is positively correlated with premature coronary artery disease (Sniderman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* *77*:604–608 (1980); Teng *et al.*, *J. Clin. Invest.* *77*:663–672 (1986)), which suggests that elevated levels of apoB-containing lipoproteins in plasma play a causal role in the development of atherosclerosis. Studies using HepG2 cells show that apoB mRNA is constitutively expressed with a relatively long half-life of 16 h (Pullinger *et al.*, *J. Lipid Res.* *30*:1065–1077 (1989)), and apoB mRNA levels do not change even in situations when apoB secretion from HepG2 cells is altered (Yao *et al.*, *J. Lipid Res.* *38*:1937–1953 (1997); Ginsberg, *Curr. Opin. Lipidol.* *6*:275–280 (1995); and Dixon *et al.*, *J. Lipid Res.* *34*:167–179 (1993)). Therefore, apoB is regulated mainly at a post-transcriptional level.

In this example, we set out to determine whether the apoB mRNA-specific hammerhead ribozyme would cleave apoB mRNA, and if this would result in decreased apoB100 mRNA levels and altered apoB production *in vivo*. To test our hypothesis, recombinant adenovirus-expressing hammerhead ribozymes targeted at nucleotide sequences $AUA^{6665}\downarrow$ and $GUA^{6679}\downarrow$ of apoB mRNA flanking the editing base C^{6666} were used to infect HepG2 cells. The results indicate that hammerhead ribozymes successfully cleave a 14-kb endogenous apoB mRNA at the expected target site. This reaction results in a reduction of apoB mRNA levels and the secretion of a truncated apoB product. Kinetic studies suggest that most of the truncated apoB was degraded intracellularly.

Ribozyme RB15 RNA cleaves apoB mRNA, generating a truncated apoB product. Pulse-chase experiments show that most of the truncated apoB product is degraded intracellularly. Thus, ribozyme treatment decreased apoB mRNA levels, decreased apoB100 production, and produced a truncated apoB that was prone to degradation. This presents a very efficient way to regulate apoB production.

Therefore, the results demonstrate the potential use of AvRB15 as a gene therapy vector to reduce atherogenic apoB-containing lipoproteins in humans.

Example II: Hammerhead Ribozyme as a Therapeutic Agent

A. Methods

Plasmid Vectors

pRB15 was used to generate synthetic ribozyme RNA (XbaI-linearized) using T7 RNA polymerase for *in vitro* ribozyme assays, or to generate the antisense ribozyme RB15 probes for the quantitation of ribozyme RB15 transcripts by the RNase protection assay. The anti-RB15 probe of 102 nucleotides was produced by HindIII-linearized pRB15 using Sp6 RNA polymerase.

pB3, containing a human apoB cDNA fragment of nucleotides 4953–5602 in pGem7Zf(+), was used to generate the antisense apoB probe of 721 nucleotides (XbaI-linearized) using T7 RNA polymerase for the quantitation of human apoB transcripts by the RNase protection assay.

pMouse-apoB, containing a mouse apoB cDNA fragment of nucleotides 6482–6762 in pGem3Zf(+), was used to generate the antisense mouse apoB probe of 283 nucleotides (HindIII-linearized) using T7 RNA polymerase for the quantitation of mouse apoB transcripts.

Recombinant Adenoviral Ribozyme Vectors

The construction of recombinant adenoviral apoB mRNA-specific hammerhead ribozymes is described in Example I. AvRB15 was designed to the target sequence of GUA↓ at nucleotide 6679 of apoB mRNA. A point mutant of the conserved catalytic domain at nucleotide $G^5 \rightarrow A$ (G5A) of RB15 is designated AvRB15 mutant, and the numbering of the conserved catalytic domain is according to Hertel *et al.*, *Nucleic Acids Res. 20*:3252 (1992). The recombinant adenoviral ribozyme cassette is driven by a Rous sarcoma virus promoter. High titer recombinant adenovirus was amplified on 293 cells and purified by $CsCl_2$ gradient centrifugation as described (Teng *et al.*, *J. Biol. Chem. 269*:29395–29404 (1994)).

Animal Experiments

Mice deficient in Apobec1 (*Apobec1*$^{-/-}$) (Nakamuta *et al.*, *J. Biol. Chem. 271*:25981–25988 (1996)) were mated with transgenic mice expressing human apoB (*ERhB*$^{+/+}$) (Callow *et al.*, *Proc. Natl. Acad. Sci. USA. 91*:2130–2134 (1994)) to generate the mouse line of *Apobec1*$^{-/-}$*ERhB*$^{+/+}$. This mouse line produces apoB100 only, with the characteristic features of elevated concentrations of apoB-containing lipoprotein particles (VLDL+LDL) and decreased concentrations of HDL compared to wild type. Recombinant adenoviral vector stock of AvRB15 or AvRB15-mutant (as control) were diluted with phosphate-buffered saline (PBS) to the appropriate concentration and $4x10^{11}$ virus particles were injected into the external jugular vein of 5–6 weeks-old mice. These mice were maintained on laboratory chow (Teklad 4% mouse/rate diet 7001). Following adenoviral vector injection, samples of blood or tissues were obtained.

Southern Blot Analysis of Liver DNA

Genomic DNA was prepared from mouse live as described (Bell *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 78*:5759–5763 (1981)). DNA was digested with BamHI under conditions recommended by the suppliers, fractionated by electrophoresis on 1% agarose gels, and transferred to a Zeta probe membrane (Bio-Rad). Filters were hybridized with ^{32}P-end labeled RB15 oligonucleotides, and radiolabeled bands were detected by autoradiogram.

Quantitation of the Levels of Ribozyme RB15 RNA and ApoB mRNA After AvRB15 Treatment

Total RNA was extracted from tissues using an Ultraspec RNA Isolation System (Biotecx Laboratory Inc., Houston, TX). The concentration of RB15 RNA and apoB mRNA in each sample was quantitated using an RNase protection RPA II kit (Ambion). Briefly, an RB15 RNA probe of 102 nucleotides was produced from the pRB15 vector (linearized with XbaI) using Sp6 RNA polymerase in the presence of ^{32}P-UTP (Amersham Pharmacia Biotech). The RNase protection assay was carried out with 10 μg total RNA and 3 x 10^4 cpm probe in 20 μl of hybridization buffer. The mixture was incubated at 45°C overnight. At the end of this reaction, the mixture was treated with 100 μl RNase digestion buffer containing RNase A and RNase T1 for 30 min at 37°C. The protected RNA fragment (59 nucleotides) was precipitated and analyzed by 8% polyacrylamide-urea gel electrophoresis and quantitated using a PhosphorImager SF scanner.

To determine the levels of human apoB mRNA after AvRB15 treatment, a ^{32}P-labeled anti-sense apoB RNA probe of 721 nucleotides was produced from the pB3 vector (linearized with XbaI) using T7 RNA polymerase in the presence of ^{32}P-UTP (10 mCi/ml, Amersham Pharmacia Biotech). The RNase protection assay was performed as described above. After RNase digestion, the protected fragment of 640 nucleotides was analyzed by 4% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner.

To determine the levels of mouse apoB mRNA after AvRB15 treatment, a ^{32}P-labeled antisense apoB RNA probe of 283 nucleotides was produced from the pMouse-apoB vector (linearized with HindIII) using T7 RNA polymerase in the presence of ^{32}P-UTP (10 mCi/ml, Amersham Pharmacia Biotech). The RNase protection assay was performed as described above. After RNase digestion, the protected fragment of 253 nucleotides was analyzed with 5% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner.

pTRI-GAPDH-mouse obtained from Ambion was used to produce a ^{32}P-labeled antisense mouse GAPDH RNA probe of 355 nucleotides (linearized with HindIII, using T7 RNA polymerase). The RNase protection assay was performed as described above. After RNase digestion, the protected fragment of 316 nucleotides was analyzed by 5% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner.

In Vitro Ribozyme Cleavage Reaction

Synthetic ribozyme RNA RB15 corresponding to antisense apoB RNA (HindIII linearized) was produced using Sp6 RNA polymerase. The concentration of synthetic ribozyme RNA was determined by measuring optical density at 260 nm. The *in vitro* ribozyme cleavage reaction was performed using mouse liver total RNA (10 µg) plus ribozyme RB15 RNA in a buffer containing 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA. The reaction was carried out for 2 h at 37°C. The RNA was precipitated, followed by Reverse Ligation-mediated PCR to detect the 3′ cleavage product.

Reverse Ligation-Mediated PCR (RL-PCR) and Sequencing

RL-PCR and sequencing was performed as described in Example I.

Lipoprotein Distribution and Analysis

Plasma levels of cholesterol and triglyceride were measured with enzymatic kits (Sigma, MO). The plasma level of human apoB100 in mouse was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Pullinger *et al.*, *J. Lipid Res. 30*:1065–1077 (1989)) with monoclonal antibody 4G3 which recognizes full-length human apoB100. The distribution of plasma lipoproteins was determined by size-fractionation of plasma (150 µl) by fast performance liquid chromatography (FPLC) using two Superose 6 columns (Amersham Pharmacia Biotech) to VLDL, LDL, and HDL. To detect human apoB100 and truncated apoB, mouse plasma (2 µl) was electrophoresed on a 6% ProSieve 50 gel (FMC BioProducts, ME), followed by Western transfer to Immunobilon P membrane (Millipore, MA). The membrane was incubated with human apoB-specific monoclonal antibody 1D1 and the immunoreactive bands were detected using an ECL system (Amersham Pharmacia Biotech). Mouse apoB100 and truncated apoB from mouse plasma were identified using mouse apoB-specific antiserum and detected using the ECL system (Amersham Pharmacia Biotech).

Statistics

Statistical comparisons were done using Statview 4.5 ANOVA.

B. Results

Hepatic Uptake and Expression of AvRB15 in Mouse Liver

Mice deficient in apoB mRNA editing enzyme (*Apobec1*$^{-/-}$) (Nakamuta *et al.*, *J. Biol. Chem. 271*:25981–25988 (1996)) were backcrossed with transgenic mice expressing human apoB (*ERhB*$^{+/+}$) (Callow *et al.*, *Proc. Natl. Acad. Sci. USA. 91*:2130–2134 (1994)) to produce the mouse line *Apobec1*$^{-/-}$*ERhB*$^{+/+}$. These mice produce full-length apoB100 only and have increased levels of plasma LDL cholesterol and apoB-containing lipoprotein particles. We injected AvRB15, AvRB15-mutant, or PBS into *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ mice via jugular veins and examined the effect of active ribozyme RB15 on apoB mRNA levels *in vivo*.

By Southern blot analysis after active ribozyme AvRB15 treatment, ribozyme RB15 DNA was detected in the liver but not in any other tissues examined. Moreover, ribozyme RB15 DNA was still readily detectable at day 35 after injection. The RNA expression of ribozyme RB15 in the liver of AvRB15-treated animals was analyzed by an RNase protection assay. Equal amounts of total RNA from different time points were used for the assay. A high level of RB15 RNA with the protected size of 60 nucleotides was found in the mouse liver of active AvRB15-treated animals and the expression of the transcripts persisted to day 35. There was no detectable RB15 transcript in AvRB15-mutant-treated animals. The results indicate that in mouse liver, the Rous sarcoma virus promoter-driven RB15 is expressed very efficiently.

Next, we examined whether the active ribozyme RB15 inhibits the expression of human apoB mRNA in *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ mice. At day 4 after active ribozyme RB15 treatment, the levels of human apoB mRNA decreased markedly by >80% compared to untreated animals of day 0 (16.7±1.3%, n=4). The lower levels of apoB mRNA persisted for many weeks. At day 21, human apoB mRNA levels were 51.4±9.7% (n=4), compared to untreated animals of day 0. By day 49, human apoB mRNA levels in mice returned to the same levels as untreated animals (101±8.9%, n=4). The apoB sequences flanking RB15 target site GUA^{6679}↓ are homologous between human and mouse (Teng *et al.*, *Biochem. Biophys. Res. Commun. 173*:74–80 (1990)). Therefore, active ribozyme RB15 cleaved mouse apoB mRNA also. The levels of mouse apoB mRNA decreased in the same fashion as that of human apoB mRNA after active ribozyme RB15 treatment. Inactive control ribozyme (RB15-mutant) had no effect on the levels of either human or mouse apoB mRNA. Thus, the results demonstrate that active ribozyme RB15 inhibited both human and mouse apoB gene expression.

To verify that RB15 ribozyme is catalytically active *in vivo*, we used the Reverse Ligation-mediated PCR (RL-PCR) method (Bertrand *et al.*, *Meth. Mol. Biol.*, Turner, P. C., ed) vol. 74, pp. 311–323, Humana Press Inc., Totowa, NJ)) to detect the 3′ ribozyme cleavage product. The cleavage product was sequenced to confirm the target site. The 65-nucleotide 3′ cleavage product was detected only in the livers of active ribozyme RB15-treated mice, but not in those of inactive ribozyme RB15-mutant-treated mice. The sequences show that active ribozyme RB15 cleaved human apoB mRNA at the expected site of nucleotide 6679. Thus, the results demonstrate that ribozyme

RB15 is catalytically active *in vivo*– it cleaves apoB mRNA at the expected site and this results in a substantial decrease in apoB mRNA levels.

To ensure that the detection of 3′ cleavage product by RL-PCR after active ribozyme RB15 treatment *in vivo* was not a result of an experimental artifact *in vitro*, we added different amounts of synthetic active ribozyme RB15 to mouse liver RNA of day 4 after inactive AvRB15-mutant treatment. There was no detectable band by RL-PCR. Therefore, the experimental procedures of RL-PCR did not produce any experimental artifact *in vitro*. To examine whether active ribozyme RB15 would cleave endogenous apoB mRNA *in vitro*, we performed *in vitro* ribozyme cleavage assays by incubating synthetic active ribozyme RB15 with mouse liver RNA of day 4 after inactive AvRB15-mutant treatment in the presence of 10 mM $MgCl_2$. By RL-PCR, a 65-nucleotide 3′ cleavage product was detected. This cleavage product was not detected in a sample not treated with synthetic active ribozyme RB15. Therefore, as demonstrated by others and here, Mg^{++} ion is essential for hammerhead catalysis (Dahm *et al.*, *Biochemistry 30*:9464–9469 (1991)). Taken together, our results demonstrate that the procedures of RL-PCR did not generate artifactual products and active ribozyme RB15 cleaved endogenous apoB mRNA.

Effect of AvRB15 Treatment on the Levels of Apolipoprotein B and Lipoproteins

To examine whether the catalytically active ribozyme AvRB15 had an effect on the production of apoB, we analyzed mouse plasma by immunoblot using human apoB-specific monoclonal antibody 1D1, which recognizes residues 474–539 (Pease *et al.*, *J. Biol. Chem. 265*:553–568 (1990)). Mouse plasma from either inactive ribozyme AvRB15-mutant- or PBS-treated animals revealed the presence of apoB100 only. In contrast, within 4 days after active ribozyme AvRB15 treatment, the levels of human apoB100 decreased and an immunoreactive truncated apoB of the expected size was detected. The same observation was shown on day 12 after active ribozyme AvRB15 treatment. Immunoblot using mouse apoB-specific antibody also detected mouse apoB100 and truncated mouse apoB in samples of day 4 after active ribozyme AvRB15 treatment, whereas only apoB100 was demonstrated in samples from either inactive ribozyme AvRB15-mutant- or PBS-treated animals. Moreover, the levels of mouse apoB100 after active ribozyme AvRB15 treatment was markedly decreased, compared to samples treated with either PBS or inactive ribozyme AvRB15-mutant. The observation of a decreased apoB level (human apoB+mouse apoB) was confirmed by SDS/PAGE

analysis with Coomassie Brilliant Blue staining. Taken together, the results indicate that active ribozyme AvRB15 cleaves apoB mRNA and results in decreased levels of full-length apoB100 and the generation of a truncated apoB of the expected size.

We next examined the effects of active ribozyme AvRB15 on plasma cholesterol, triglyceride, and human apoB in mice. On day 7 after inactive AvRB15-mutant ribozyme injection, the levels of plasma cholesterol, triglyceride, and human apoB increased 30% (n=12), 58% (n=12), and 56% (n=13), respectively, compared to day 0 before treatment. In contrast, active ribozyme AvRB15 treatment attenuated the levels of plasma cholesterol, triglyceride, and human apoB to 24% (n=18), 22% (n=16), and 40% (n=19), respectively, compared to day 0 before treatment. When compared that to the inactive AvRB15-mutant treated group, the active AvRB15-treated group displayed decreases in plasma cholesterol, triglyceride, and human apoB of 42%, 51%, and 62%, respectively. These markedly decreased levels persisted to day 21, returning toward pretreatment levels by day 35. There was no change in any of the parameters measured in animals treated with PBS throughout this study.

Plasma from either active ribozyme AvRB15-treated or inactive ribozyme AvRB15-mutant-treated mice of day 0 before treatment and of days 7, 14, 21, 35, and 49 after treatment was fractionated into VLDL, LDL, and HDL fractions on an FPLC column. The expression of active ribozyme AvRB15 in mouse liver had a profound effect on the distribution of plasma lipoproteins. At day 7 after mice were treated with active ribozyme AvRB15, the cholesterol concentrations in the VLDL and LDL fractions were barely detectable, compared to that of inactive ribozyme AvRB15 mutant-treated animals. This pattern of reduced cholesterol levels continued to days 14 and 21. By day 35, the lipoprotein profile returned to the same pattern as that of day 0 before treatment. In comparison to day 0 before treatment, the cholesterol in the HDL fractions from both groups was decreased.

To examine the distribution of truncated apoB in lipoprotein fractions, we pooled and concentrated VLDL, LDL, and HDL fractions, and analyzed the pooled fraction by immunoblot using monoclonal antibody 1D1. Human apoB100 was detectable in all the major lipoprotein fractions including VLDL, LDL, and HDL, but its concentration was highest in the LDL fraction. Interestingly, the truncated apoB was also detected in VLDL, LDL, and HDL fractions. By scanning the immunoblot with a

densitometer, we found that at day 7 after active ribozyme RB15 treatment, the ratio of truncated apoB to apoB100 was ~50%, 10%, and 65% in VLDL, LDL, and HDL fractions, respectively. From days 14 to 21, the levels of truncated apoB decreased gradually in both VLDL and LDL fractions, while that in HDL fraction remained the same. Interestingly, the full-length apoB100 was almost undetectable in the HDL fraction during this period of time.

C. Discussion

In the present study we used a novel approach to lower plasma LDL levels and to prevent the initiation of the atherosclerotic process. The objective was to downregulate apoB mRNA by the *in vivo* delivery of a specific hammerhead ribozyme directed against the mRNA. The striking reduction of apoB mRNA levels after active ribozyme RB15 treatment is exciting, since apoB mRNA levels typically do not change in response to a variety of metabolic interventions (Young, *Circulation 82*:1574–1594 (1990)). The greater than 80% reduction in apoB mRNA levels led to a comparable decrease in apoB100 levels (~61%), which represents a very efficient way to regulate apoB production. Moreover, the ribozyme RB15 treatment resulted in the production of a truncated apoB of the predicted size. Our study in HepG2 cells using pulse-chase experiments suggests that, compared with full-length apoB100, most of the truncated apoB was degraded intracellularly (only ~30% of the truncated apoB radioactivity was recovered in the media).

There was a marked reduction of apoB-containing lipoproteins in animals treated with AvRB15. Therefore, using ribozyme targeted at apoB mRNA *in vivo*, we demonstrated that AvRB15 can be used potentially as an effective gene therapy vector to reduce atherogenic apoB-containing lipoproteins in humans. Furthermore, the generation of truncated apoB mimics the phenotype of hypobetalipoproteinemia. Since ribozyme targets at the levels of apoB mRNA, it does not affect the genomic DNA, and the production of apoB mRNA-specific ribozyme transgenic mice would not be embryonic lethal as demonstrated in homozygotes when mouse apoB genes were disrupted (Kim *et al.*, *J. Clin. Invest. 101*:1468–1477 (1998); Farese *et al.*, *Proc. Natl. Acad. Sci. USA. 92*:1774–1778 (1995); and Homanics *et al.*, *Proc. Natl. Acad. Sci. USA. 90*:2389–2393 (1993)). An apoB mRNA-specific ribozyme transgenic mouse should be a useful animal model for studying hypobetalipoproteinemia.

It has previously been demonstrated that adenovirus-mediated gene transfer of *Apobec1*, the apoB mRNA editing enzyme, in mice or rabbits decreases the levels of plasma cholesterol, apoB, and LDL particles (Teng *et al.*, *J. Biol. Chem. 269*:29395–29404 (1994); Kozarski *et al.*, *Human Gene Therapy 7*:943–957 (1996); Hughes *et al.*, *Human Gene Therapy 7*:39–49 (1996); Teng *et al.*, *Arteriosclerosis, Thrombosis, and Vascular Biology 17*:889–897 (1997); and Greeve *et al.*, *J. Lipid Res. 37*:2001–2017 (1996)). *Apobec1* has no effect on the levels of apoB mRNA; it decreases apoB100 levels by increasing the conversion of apoB100 to apoB48. The fate of apoB48 is similar to the truncated apoB generated by AvRB15 treatment. A study demonstrated that *LDLR*$^{-/-}$*Apobec1*$^{-/-}$ ice developed much more extensive lesions than those in *LDLR*$^{-/-}$*Apobec1*$^{+/+}$ mice (Powell-Braxton *et al.*, *Nature Med. 4*:934–938 (1998)). Therefore, apoB mRNA editing limits the accumulation of LDL. In the present study, by using a ribozyme targeted at apoB mRNA, we produced a marked reduction in the apoB mRNA levels which, in turn, decreased plasma lipid and apoB100 concentration. Therefore, this ribozyme technology should be a useful adjuvant to LDL receptor gene therapy for the treatment of familial hypercholesterolemia.

All references cited herein are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the art that the invention may be performed within a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

What is Claimed is:

1. A ribozyme that specifically hybridizes to and cleaves apoB100 mRNA, wherein said ribozyme consists essentially of:
   (a) a stem loop region 12 to 40 bases in length that forms a stem loop conformation when said ribozyme hybridizes to apoB100 mRNA;
   (b) a target segment three bases in length, wherein:
      (i) said target segment is located within said stem region; and
      (ii) said target segment has a sequence consisting of NUX, wherein N represents any base, and X is A, C, or U;
   (c) two flanking segments 8 to 50 bases in length, wherein:
      (i) each flanking segment has a sequence complementary to a corresponding sequence in apoB100 mRNA; and
      (ii) one flanking segment lies immediately 3' to said stem loop region and the other lies immediately 5' to said stem loop region.

2. The ribozyme of claim 1, wherein said ribozyme cleaves apoB100 mRNA specifically at position 6679.

3. The ribozyme of claim 2, wherein said ribozyme has the target sequence GUA and wherein one flanking segment comprises a sequence consisting essentially of SEQ ID NO: 1 and the other flanking segment comprises a sequence consisting essentially of SEQ ID NO:2.

4. The ribozyme of claim 1, wherein said ribozyme has a sequence consisting essentially of SEQ ID NO:3.

5. A DNA molecule consisting essentially of nucleotides encoding the ribozyme of any one of claims 1-4.

6. An expression vector comprising the DNA of claim 5.

7. The vector of claim 6, wherein said vector is a retroviral vector.

8. The vector of claim 7, wherein said vector is an adenoviral vector.

9. A host cell transformed with the vector of claim 6.

10. A method of reducing the concentration of apoB100 in the plasma of a patient, comprising administering to said patient the expression vector of claim 6.

11. The method of claim 10, wherein said expression vector is a retroviral vector.

12. The method of claim 11, wherein said vector is an adenoviral vector.

13. The method of claim 10, wherein said patient is treated for hypercholesterolemia.

14. A mouse that serves as a model of hypobetalipoproteinemia, wherein said mouse is administered the vector of claim 6 at a concentration sufficient to significantly reduce the concentration of apoB100 in the plasma of said mouse compared to the concentration present prior to administration.

15. The mouse of claim 14, wherein said vector is a retroviral vector.

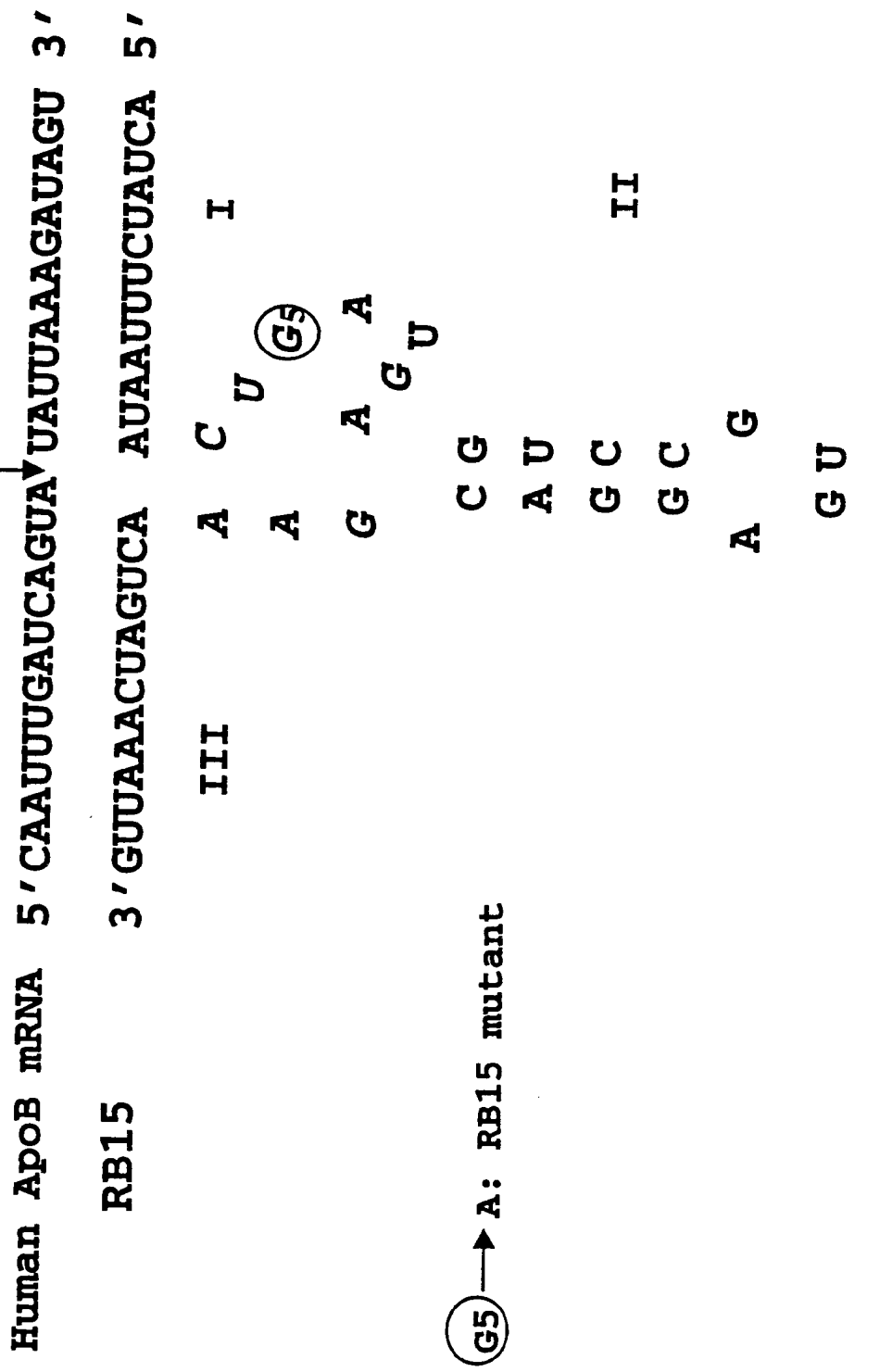
16. The mouse of claim 15, wherein said vector is an adenoviral vector.

17. The mouse of claim 14, wherein said mouse is a transgenic mouse.

18. The transgenic mouse of claim 17, wherein said transgenic mouse has the *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ phenotype.

19. A transgenic mouse engineered to constitutively express the ribozyme of any one of claims 1-4.

20. The transgenic mouse of claim 19, wherein said mouse has the *Apobec1*$^{-/-}$*ERhB*$^{+/+}$ phenotype.


6679
Human ApoB mRNA 5′CAAUUUGAUCAGUA▼UAUUAAAGAUAGU 3′
RB15 3′GUUAAACUAGUCA AUAAUUUCUAUCA 5′
III
A C U
I
A
G A A
G U
G5
C G
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G C
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II
G5 → A: RB15 mutant

Figure 1

SEQUENCE LISTING

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Teng, Ba-Bie
Tiebel, Margret

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 00/22481

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/00 C12N5/10 A61P3/06 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61P A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WANG ET AL: "Apolipoprotein B specific hammerhead ribozyme results in production of truncated apolipoprotein B in cultured human hepatoma cells" CIRCULATION, vol. 94, no. 8, 15 October 1996 (1996-10-15), page I631 XP000981241 abstract ---	1-20
Y	ENJOJI ET AL: "Apolipoprotein B mRNA specific hammerhead ribozyme reduces apolipoprotein B and plasma cholesterol levels in mice" CIRCULATION, vol. 98, no. 17, 1998, pages I2-I3, XP000981225 abstract --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2001	02. 3. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Korsner, S-E

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/22481

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 09392 A (RIBOZYME PHARMACEUTICALS) 28 March 1996 (1996-03-28) the whole document	1-20
Y	BIRIKH K R ET AL: "The structure, function and application of the hammerhead ribozyme" EUROPEAN JOURNAL OF BIOCHEMISTRY,DE,BERLIN, vol. 245, no. 1, 1 April 1997 (1997-04-01), pages 1-16, XP002045195 ISSN: 0014-2956 the whole document	1-20
A	WO 99 35257 A (HAROSH) 15 July 1999 (1999-07-15) figure 4	1-20
P,X	WANG ET AL: "Hammerhead ribozyme cleavage of apolipoprotein B mRNA generates a truncated protein" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 34, 20 August 1999 (1999-08-20), pages 24161-24170, XP002159202 the whole document	1-20

9

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 00/22481

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10-13
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Although claims 10-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.ernational application No.
PCT/US 00/22481

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 10-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 10-13

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte: ›nal Application No
PCT/US 00/22481

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9609392 A	28-03-1996	US 5599706 A	04-02-1997
		AU 3720295 A	09-04-1996
		CA 2199727 A	28-03-1996
		EP 0782622 A	09-07-1997
		JP 10506016 T	16-06-1998
		US 5877022 A	02-03-1999
WO 9935257 A	15-07-1999	FR 2773079 A	02-07-1999
		AU 1971399 A	26-07-1999
		EP 1042463 A	11-10-2000